Cytochemistry of the Tergite Epicuticle of *Glomeris marginata* (Villers) (Myriapoda, Diplopoda): Preliminary Experimental Results

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ABSTRACT

The present study determines the ultrastructural location of chitin, proteins, and lipids in the epicuticle of the diplopod *Glomeris marginata* (Villers). The results lead to the conclusion that the cuticle includes two functionally different parts: the upper part is involved in the permeability of the cuticle whilst the lower has mainly a mechanical role. The upper part includes three epicuticular layers probably homologous to those described in insects: the cuticulin layer, the wax layer, and the proteinaceous cement layer. The former seems to consist of a median leaflet of stabilized lipid polymers sandwiched in two protein leaflets. This arrangement is assumed to be a primitive, general feature of the arthropod cuticle, having been identified as the main waterproofing barrier in the cuticle of marine decapod crustaceans. The inner epicuticle and the mineralised procuticle play a mechanical role. The inner epicuticle consists of a lipoprotein matrix that surround rod-shaped protein elements and chitin-protein fibres which are probably of procuticular origin. Structurally and functionally, it might be regarded as a structure convergent with that of decapod crustaceans, that plays a part as a reinforcement to prevent the epicuticle splitting off from the mineralised exocuticle.

RÉSUMÉ

Etude cytochimique de l'épicuticule des tergites de *Glomeris marginata* (Villers) (Myriapoda, Diplopoda).

Le présent travail concerne la localisation ultrastructurale de la chitine, des protéines et des lipides de l'épicuticule du diplopode *Glomeris marginata* (Villers). D'un point de vue fonctionnel, les résultats amènent à conclure que la cuticule comprend deux parties différentes : la partie supérieure intervient dans la perméabilité de la cuticule tandis que la partie inférieure joue un rôle essentiellement mécanique. La partie supérieure comporte trois couches épicuticulaires de surface, probablement homologues de celles décrites chez les insectes : la couche de cément protéinique, la couche de cire, et la cuticuline. Cette dernière semble former un feuillet médian de polymères lipidiques stables pris en sandwich entre deux feuillets protéiques. On admet que la cuticuline est un constituant primitif commun à toutes les cuticules d'arthropodes. Elle est reconnue comme la principale barrière imperméable de la cuticule des crustacés décapodes marins. Les couches jouant un rôle mécanique sont l'épicuticule interne et la procuticule minéralisée. L'épicuticule interne consiste en une matrice lipoprotéique entourant des éléments protéiques en forme de batonnets et des fibres chitinoprotéiques d'origine probablement procuticulaire. D'un point de vue structural et fonctionnel, cette constitution de l'épicuticule interne peut être considérée comme une convergence avec celle rencontrée chez les crustacés décapodes, en raison de son rôle de renfort empêchant l'épicuticule de se détacher de l'exocuticule minéralisée.

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INTRODUCTION

The arthropod cuticle is commonly regarded as an outer integumental structure acting as an exchange surface in addition to providing mechanical protection. From an adaptive and evolutionary point of view, the differentiation of several epicuticular layers with peculiar structures and chemical composition has probably contributed to the success of this group in colonising a wide variety of environments.

As a result, the layers of epicuticle show a high degree of specialisation according to the physiology of the integumental regions and to the habitat. For instance, it is well known that cuticular lipids such as the surface waxes represent a barrier against water loss in terrestrial arthropods such as insects and arachnids (HADLEY, 1981). In this respect, diplopods appear as a very original and interesting group to study, since they are phylogenetically close to insects but live in nearly the same wet microhabitats as terrestrial isopods and possess a mineralised cuticle as it is the rule in numerous crustaceans. Little is known about the structure and especially the chemical composition of the epicuticular layers.

Recent ultrastructural observations of ANSENNE *et al.* (1990), have shown that the organisation of the cuticle of *Glomeris marginata* (Villers) fits the classical scheme known from arthropods. In both the cuticle consists of a thin epicuticle overlying a thick, lamellate procuticle which is subdivided into an exocuticle and an endocuticle. The cuticle is traversed by pore canals and ducts of dermal glands. However, the epicuticle exhibits peculiar features whose interpretation is critical (Fig. 1). The outermost epicuticular layers of diplopods with its cuticulin, wax and cement layers are nearly identical in appearance to those of insects, but have never been clearly identified. In addition, the structure of the inner epicuticle appears to be very peculiar because microfibre-like elements that are arranged in a twisted plywood structure are embedded in a matrix of medium electron density. Consequently, these structural peculiarities raise important questions about the chemical nature of the epicuticle layers of diplopods in relation to their roles in integument physiology and to their possible degree of homology or analogy to corresponding structures in other arthropods.

The main purpose of this study was to determine the chemical nature of the epicuticle components in the tergites of *G. marginata*, using cytochemical methods for the ultrastructural demonstration of chitin, proteins, and lipids. The results are discussed with special reference to the identity of the layers, their role in waterproofing or cuticle hardening, and their comparison with the cuticles of terrestrial and aquatic arthropods.

MATERIAL AND METHODS

Individuals of *Glomeris marginata* (Villers) were collected on the University campus of the Sart Tilman, Liège. To demonstrate chitin, ultrathin sections of glutaraldehyde-fixed, EDTA-decalcified and epoxy-resin-embedded tergites were incubated for 45 min on drops of a WGA-BSA-gold complex (wheat germ agglutinin, Sigma) in 0.02 M Na-phosphate buffer, pH 7.2 containing 0.5% BSA (HORISBERGER & ROSSET, 1977).

Tannic acid in the fixative medium was used as an indicative reagent for proteins (Hayat, 1993). EDTA-demineralised tergites were first fixed for 2 h at 20°C in a mixture of 1% tannic acid and 2.5% glutaraldehyde in 0.1 M Na-phosphate buffer pH 7.4, followed by a 72-hours incubation in 1% tannic acid. Protein-bound tannic acid was then revealed by "en bloc" uranyl acetate staining.

Two methods based on the reduction of OsO_4 were used to demonstrate lipids. The first was to increase the specificity of OsO_4 for unsaturated bonds in lipids under controlled experimental conditions (WIGGLESWORTH, 1981) and blocking reactions. Prior to staining (1 h at 20°C in 1% OsO_4 in 0.1 M Na-phosphate buffer, pH 7.4) the glutaraldehyde-fixed and EDTA-demineralised samples were treated for 4 h at 37°C in 1.25% N-ethylmaleimide-buffered solution for blocking sulfhydryl groups (GABE, 1968), then for 16 h at 20°C in nitrous acid (LILLIE, 1954 *in* GABE, 1968) or for 72 h in 2.5% glutaraldehyde fixative solution for blocking primary amines and then for 1 h at 60°C in saturated bromine water for blocking unsaturated bonds (MUKHERJI *et al.*, 1960). Free and bound lipids were distinguished after extraction of free lipids from glutaraldehyde-fixed material in a hot chloroform/methanol mixture.

The second method was detection of hydrophobic substances. A highly unsaturated lipid-soluble marker, myrcene, was incorporated by partition in 50% ethanol, then revealed by reduction of OsO_4 (WIGGLESWORTH, 1981). This treatment was performed after bromination.

Source : MNHN, Paris

RESULTS

The WGA-BSA-gold complex which is used to demonstrate chitin labels the microfibres in the procuticle and inner epicuticle (Fig. 2). In both layers, labelling depends on microfibre orientation as determined by the twisted plywood arrangement of the microfibres. The gold particles are distributed only along successive horizontal bands where the microfibres are seen in oblique section and are absent where the fibres appear in longitudinal section.

The method used to demonstrate proteins, tannic acid treatment followed by "en bloc" uranyl acetate staining, strongly enhances the electron density of the whole procuticle and of the cement layer, the cuticulin layer, and the inner epicuticle (Figs 3 & 4). In contrast, the wax layer remains completely electron-lucent. In the inner epicuticle, electron-dense rod-shaped elements are prominent against the electron lucent material of the matrix. As observed after classical staining (Fig. 1), these elements are oriented parallel to the microfibres.

The first method of lipid demonstration reveals unsaturated lipids in different epicuticular layers and shows that they are insoluble in organic solvents (Figs 5 & 6). The lipids are mainly located in the wax layer but also impregnate the cement layer and the upper leaflet of the cuticulin layer, both of which appear as intensely electron-dense borders. The moderate electron density of the lower leaflet of the cuticulin layer and inner epicuticle indicates that these layers are relatively poor in such lipids. As a control, oxidation of double bonds by bromination prior to OsO_4 staining prevents any osmiophilic reaction in the wax layer but merely reduces the contrast in the other epicuticular layers (Fig. 7). This remaining osmiophily is probably due to the presence of proteins. The presence of lipids in the epicuticular layers of *G. marginata* is confirmed by the results of the second procedure for detecting hydrophobic substances. Incorporation of myrcene after bromination and before OsO_4 staining restores a high electron density in the previously osmiophilic layers, i.e. the cement layer, the wax layer, and the upper leaflet of the cuticulin layer (Figs 8 & 9). However, it only slightly enhances the contrast of the lower leaflet of the cuticulin layer and the matrix material of the inner epicuticle, which seem to consist of lipoproteins. In contrast, the rod-shaped protein elements and the chitin microfibres remain electron-lucent.

DISCUSSION

The present cytochemical results combined with the previous ultrastructural observations of ANSENNE *et al.* (1990) allow accurate identification of the structural components of *Glomeris marginata* tergite epicuticle. Furthermore, considering the respective structures, chemical compositions, and roles of its constituents, the cuticle of *G. marginata* is consistent with the functional model recently proposed by COMPÈRE & GOFFINET (1992) for decapod crustaceans. According to this model, the cuticle includes two functional parts: the upper part is responsible of the integument permeability characteristics while the lower part contributes to the mechanical resistance of the exoskeleton. In *G. marginata,* the upper part includes the three outer epicuticular layers, i.e. the cement layer, the wax layer, and the cuticulin layer. The cement and wax layers can be regarded as integument adaptations to a terrestrial mode of life and as structures homologous to the corresponding layers of insect and arachnid cuticles (NEVILLE, 1975; FILSHIE, 1976; HADLEY, 1986). This view is strongly supported by their structure, location, chemical composition, and role in cuticular waterproofing in addition to the fact that the cement layer of *G. marginata* is discharged on the cuticular surface by dermal gland ducts (ANSENNE *et al.*, 1990), similar as in insects.

Since it labels both protein- and lipid-positive, the cement layer appears as an outer protection layer made of wax-impregnated proteins. Overlying the cuticulin layer, the wax layer consists exclusively of stabilized lipid compounds, most of them unsaturated. Similar solvent-resistant waxes have been reported for some insects (WIGGLESWORTH, 1985), for some arachnids (HADLEY, 1981), and for the diplopod *Orthoporus ornatus* (Girard) (WALKER &

Source : MNHN, Paris

CRAWFORD, 1980). More recently, the presence of a strongly osmiophilic wax layer was also described in *Ophyiulus pilosus* (Newport) (THOREZ *et al.*, 1992). The higher resistance of *G. marginata* to a dry environment compared to the poor water resistance of the isopod *Oniscus asellus* (L.) (EDNEY, 1951), could be ascribed to the absence of this layer in *O. asellus*, which only possesses free lipids inside the cuticulin layer instead of a distinct outer wax layer (COMPÈRE, 1990).

Although the accurate identification of the cuticulin layer components is still lacking, the present observations combined with those of ANSENNE *et al.* (1990) strongly suggest that the structure of this layer is comparable to that described for other arthropods (insects: LOCKE, 1966; arachnids: FILSHIE, 1976; crustaceans: COMPÈRE, 1988, 1990). The cuticulin layer of *G. marginata* exhibits a membrane-like structure approximately 20 nm thick which seems to consist of two protein leaflets, the upper one being impregnated with overlying waxes. These leaflets are separated by an electron-lucent layer probably made of lipid polymers, as suggested by the studies of WIGGLESWORTH (1985) and HACKMAN (1986) for insects and COMPÈRE & GOFFINET (1992) for the crab *Carcinus maenas* (L.). Appearing as a general arthropodian feature, the cuticulin layer can be regarded as a primitive structure, since it already constitutes the main permeability barrier of the cuticle in marine crustaceans (COMPÈRE & GOFFINET, 1992).

The inner epicuticle and procuticle are layers of the lower part which probably contribute to the mechanical properties of the cuticle. The inner epicuticle consists of a lipoprotein matrix surrounding proteinaceous rod-shaped elements and chitin-protein microfibres that prolong the helicoidal twisted plywood arrangement of the exocuticle fibres. This organisation appears very peculiar, never having been reported before in any other arthropod cuticle. The presence of chitin-protein fibres contradicts the first and classical definition of the insect epicuticle as a non-chitinous layer (KÜHNELT, 1928a,b). Considering that the inner epicuticle of the soft intersegmental regions of *G. marginata* (COMPÈRE, unpublished results) is composed, as in insect tergites (LOCKE, 1969; NEVILLE, 1975), of a homogeneous fibreless matrix, the thick inner epicuticle of the mineralised tergites of *G. marginata* can be interpreted as a result of close interpenetration between the epicuticular matrix material and the chitin-protein fibres of the exocuticle. Functionally, it can be regarded as a structure convergent with the inner epicuticle of decapod crustaceans, in addition to other features such as the pseudo-reticulate pattern of the exocuticle and mineralisation of the procuticle (ANSENNE *et al.*, 1990). As proposed by COMPÈRE & GOFFINET (1992) for the crab *C. maenas*, the thick fibrous inner epicuticle bearing roots on its lower side ensures mechanical reinforcement, preventing the upper layers and the mineralised exocuticle from splitting off.

FIG. 1. — Transverse section through the tergite epicuticle of *Glomeris marginata* after classical tissue fixation and uranyl acetate/lead citrate section staining. c, cuticulin layer; ct, cement layer; ie, inner epicuticle; rs, rod-shaped elements; w, wax layer;

➤, cross-sectioned chitin-protein microfibres; ❍/≡/⊗, microfibre orientation. Bar 5 µm.

FIG. 2. —Transverse section of the tergite epicuticle of *Glomeris marginata* after incubation with the WGA-BSA-gold complex and stained in uranyl acetate. ex, exocuticle; ie, inner epicuticle; ❍/≡/⊗, fibre orientation. Bar 0.5 µm.

FIGS 3 & 4. — Detail of the upper epicuticular layers after "en bloc" uranyl acetate staining (FIG. 4) and after exposure to tannic acid prior to "en bloc" uranyl acetate staining (FIG. 3). c, cuticulin layer; ct, cement layer; ie, inner epicuticle; rs, rod-shaped elements; w, wax layer. Bars 250 nm.

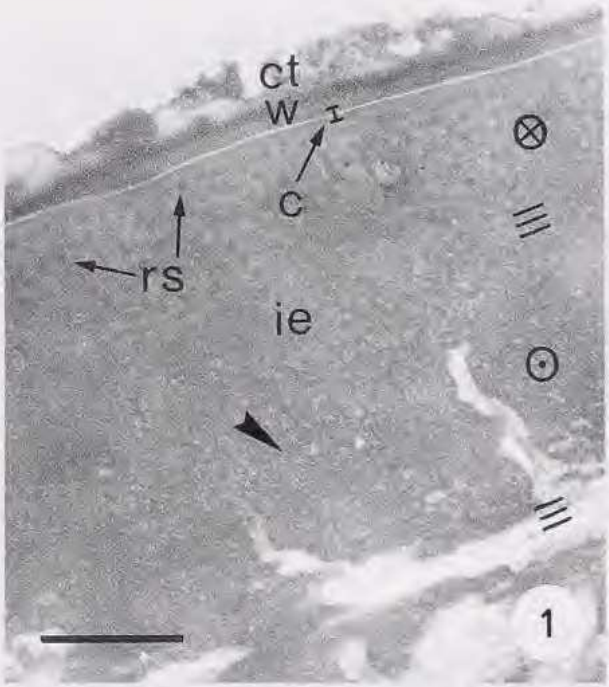
FIGS 5-9. — Vertical sections of the tergite epicuticle of *Glomeris marginata* after different cytochemical treatments. c, cuticulin layer; ct, cement layer; ex, exocuticle; ie, inner epicuticle; rs, rod-shaped elements; w, wax layer.

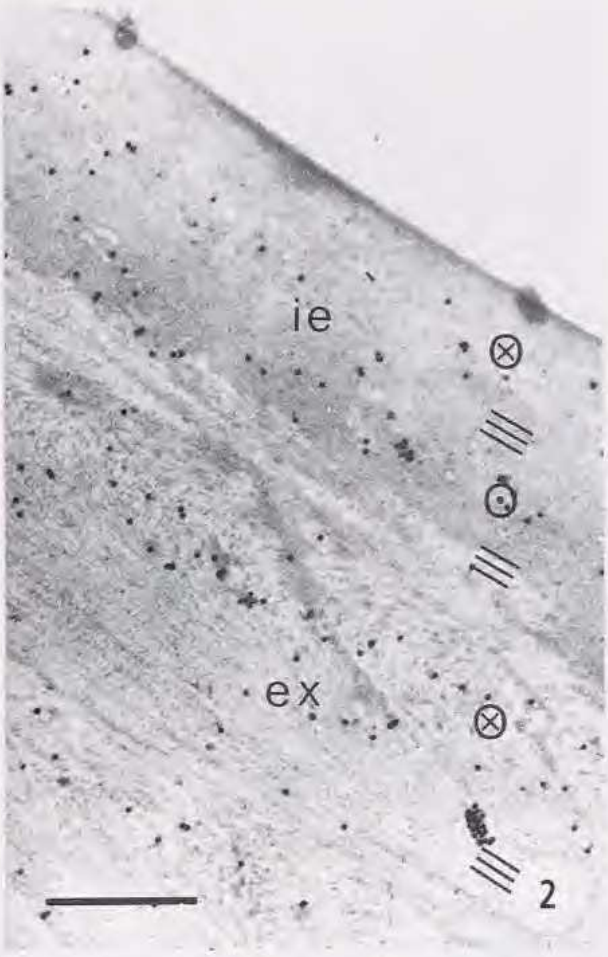
FIGS 5-6. After extraction of free lipids in a chloroform/methanol mixture, long fixation in buffered 2.5% glutaraldehyde and OsO_4 staining. Fig 5. Bar 1 µm. FIG. 6. Detail of the upper epicuticular layers. Bar 250 nm.

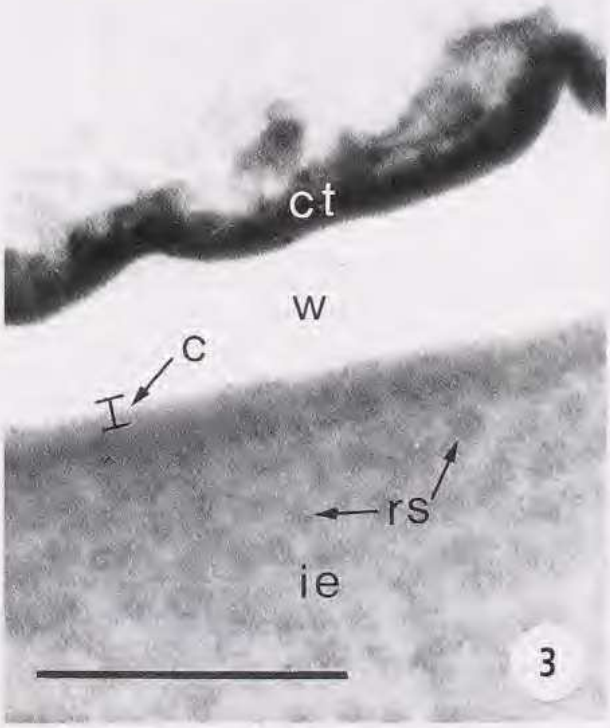
FIG. 7. Detail of the upper epicuticular layers after extraction of free lipids in a chloroform/methanol mixture, incubation in bromine water and OsO_4 staining. Bar 250 nm.

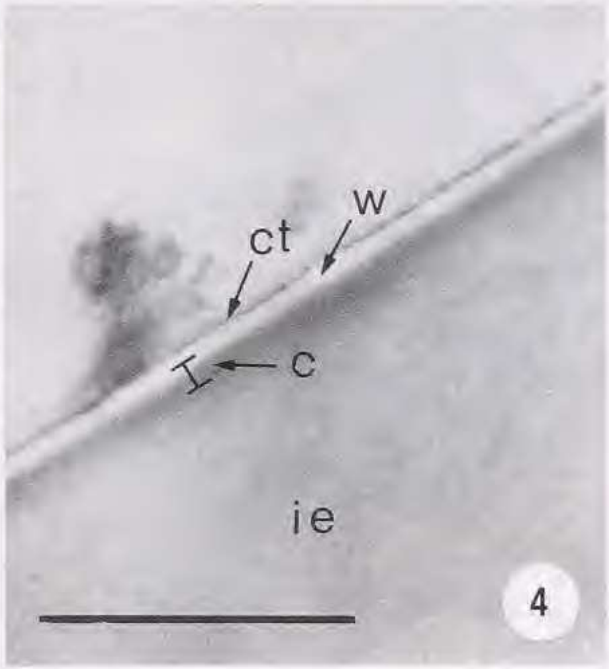
FIGS 8-9.— After extraction of free lipids in a chloroform/methanol mixture, incubation in bromine water, exposure to myrcene and OsO_4 staining. FIG. 8. Bar 1 µm. FIG. 9. Detail of the upper epicuticular layers. Bar 250 nm.

Source : MNHN, Paris









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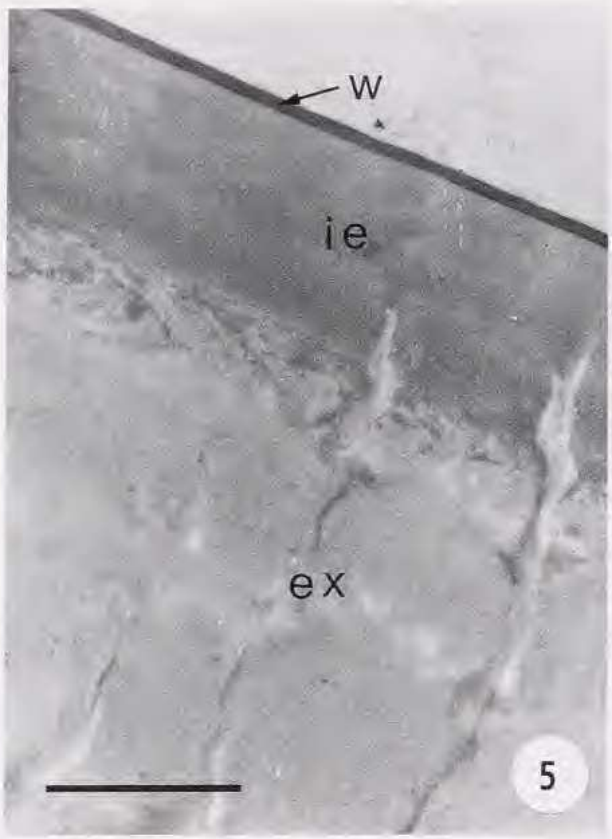

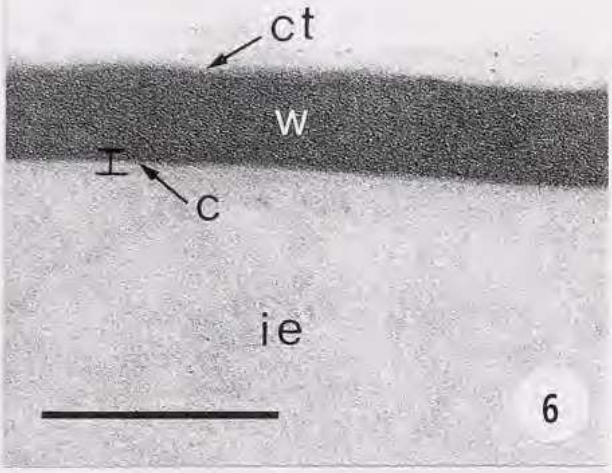

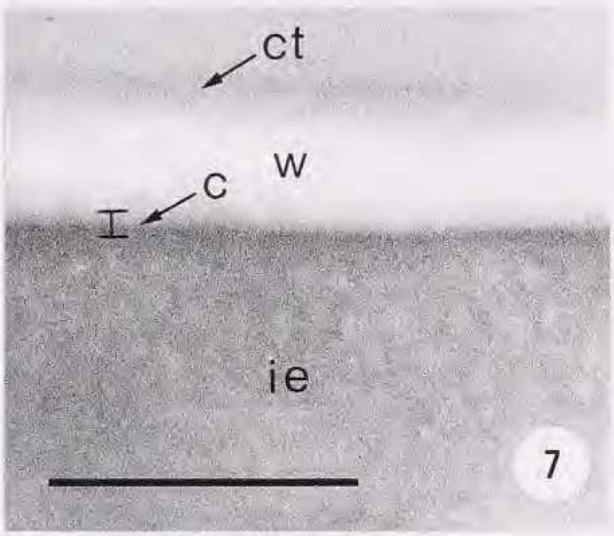

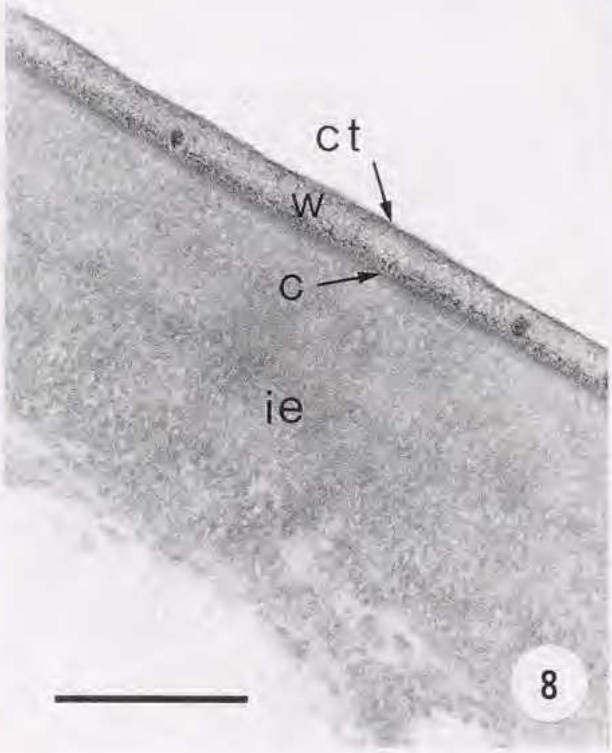

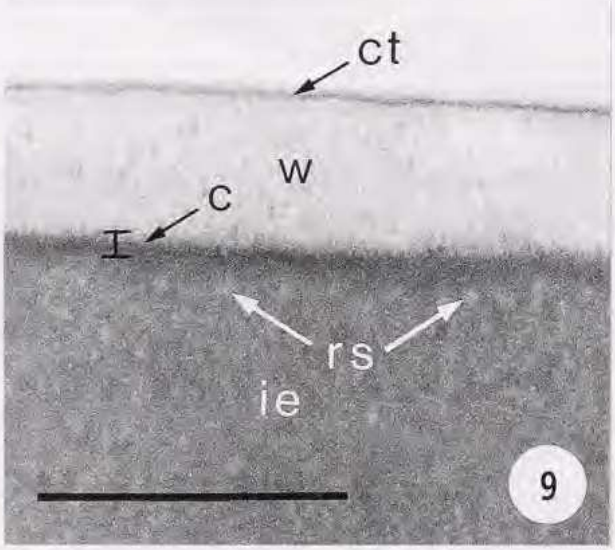

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CONCLUSION

In conclusion, the present study sheds some light upon the relationships between the different cuticle layers in diplopods, their role in integumental physiology, and their ecological significance. In addition, our evidence supports the dual-function model of the cuticle, recently defined by COMPÈRE & GOFFINET (1992) for a marine decapod crustacean. This model also agrees with previous observations made on the cuticle of insects and arachnids (for reviews, see: NEVILLE, 1975; HADLEY, 1981, 1984, 1986), we tentatively propose it as more general for arthropod cuticular structure.

ACKOWNLEDGEMENTS

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Source : MNHN, Paris